The Glucocorticoid-Induced TNFR-Related protein (GITR) contributes to the systemic adjuvanticity of the *Escherichia coli* heat-labile enterotoxin

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Keywords: T Cells, costimulation, vaccination

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Abbreviations used in this article: LT: *Escherichia coli* heat-labile enterotoxin; **CT:** *Vibrio cholerae* toxin; **GITR:** glucocorticoid-induced TNFR-related protein; **GITR$^{-/-}$:** GITR-deficient; **hBcl-2:** human Bcl-2; **B6:** C57BL/6; **FADD-DN:** dominant negative form of FADD; **Alum:** aluminium hydroxide; **DHGG:** monomeric deaggregated human gammaglobulin; **AHGG:** aggregated HGG; **f.p.:** footpad;

Summary

The *Escherichia coli* heat-labile enterotoxin (LT) possesses a powerful mucosal and systemic adjuvant effect. However, if little is known about the cellular and molecular basis of the immunostimulatory activity of LT at the mucosal level, even less information is available on the mechanisms underlying its systemic adjuvant activity. Here, we show that distinct mechanisms are responsible for the parenteral and mucosal adjuvanticity of LT. Indeed, the systemic administration of LT upregulates the expression of GITR, but not other activation markers, in naïve T cells. Using wild type and GITR-deficient mice and LT and its enzymatically inactive mutant LTK63 as adjuvants, we show that the induction of GITR expression in T cells accounts for the systemic immunostimulatory capacity of LT which requires an intact enzymatic activity. In contrast, the mucosal administration of LT does not induce GITR expression on Peyer's Patches T cells and accordingly no differences are observed in the mucosal adjuvanticity of LT between wild type and GITR-deficient mice. Altogether, our results demonstrate the distinct effect of LT after parenteral administration as compared to the mucosal delivery, and describe a new mechanism of LT adjuvanticity related to its ability to induce the expression of GITR in $CD4^+$ T cells.

Introduction

The *Escherichia coli* heat-labile enterotoxin (LT) and *Vibrio cholerae* toxin (CT) are two bacterial toxins with a powerful adjuvant capacity [1]. Both toxins consist of a monomeric A subunit with enzymatic activity and of a homo-pentameric ring of B subunits which binds to the cell surface ganglioside GM1. The enzymatic activity of these toxins results in a persistent activation of adenylate cyclase and the increase of cytosolic cAMP [2], which disrupts cell homeostasis with a variety of toxic effects [2, 3]. The high toxicity of LT and CT has stimulated the search for mutants with low or no toxicity retaining their immunostimulatory capacity. The LTK63 (Ser to Lys substitution at position 63 of the A subunit of LT) and LTR72 (Ala to Arg substitution at position 72 of the A subunit of LT) toxoids are two LT mutants with none or a reduced enzymatic activity and toxicity, respectively [4, 5]. However, the attenuation of the enzymatic activity of these mutants is associated with some decrease in their adjuvanticity [4, 5]. Thus, it becomes important to understand the mechanisms that link enzymatic activity and adjuvanticity of LT. Most of the present knowledge of the adjuvant effects of these molecules is based on experiments with animals immunized mucosally, mainly i.n. [6]. However, some reports from animal [7] and human studies [8, 9] have raised concerns on the safety of these molecules after i.n. administration. LT and LT mutants are also strong adjuvants following parenteral administration [10, 11]. However, if little is still known on the fine molecular mechanisms underlying the mucosal adjuvanticity of LT and LT mutants, even less information is available on the effects triggered by these molecules after systemic administration.

Several studies point to DC as the principal cellular target of LT and CT adjuvanticity *in vivo* [12, 13]. Both toxins induce the maturation of DC, increasing their

antigen presentation capability, their migration to the lymph nodes and their interaction with naïve T cells [14]. In addition, CT and LT may act directly on lymphocytes inducing their activation and/or cell death [3, 15, 16]. We have previously shown that administration of LT promotes an intense but incomplete apoptosis of lymphocytes [5, 16]. This, together with the capacity of the toxin to stimulate the remaining lymphocytes [15], may explain the apparent paradox that an agent which such a potent pro-apoptotic activity can be an adjuvant. An interesting observation is that the LT-mediated cell death is only observed after its systemic administration [3], suggesting that the immunomodulatory properties of LT are highly influenced by the route of toxin delivery. Because of the glucocorticoid-mediated effect of LT on apoptosis [3, 16], our interest was focused on the glucocorticoid-induced TNFR-related protein (GITR), a member of the TNFR superfamily that is constitutively expressed at high levels in Tregs and at low levels on conventional $CD4^+CD25^-$ and $CD8^+$ T cells where it is rapidly up-regulated after activation [17]. Since GITR-GITRL signalling pathways can influence the activation and activity of effector and regulatory T cells as well as DC [17-19], we have analyzed the possible role of GITR in the systemic or mucosal adjuvanticity of LT in comparison to the enzymatically inactive LTK63 mutant. We report here that the systemic adjuvanticity of LT associated with the enzymatic activity is linked to their capacity to induce the expression of GITR in T cells. We further demonstrate that the mechanisms of adjuvanticity of these immunostimulatory molecules can be distinct at different anatomical sites.

Results

Systemic, but not mucosal, administration of LT induces the expression of GITR in naïve T cells.

The expression of a panel of markers associated with lymphocyte activation was explored in mature T and B lymphocytes 72 hrs after systemic LT administration. Injection of 1 μg of LT into the footpad (f.p.) of BALB/c mice caused a marked reduction in the number of total $CD4^+$ cells in the spleen and of $CD4^+$ T cells that were either $CD25^+$, $CD69^+$, CTLA-4^+, $CD45RB^{-/low}$ or $CD62L^-$ (Figure 1A). Remarkably, the number of $CD4^+$ T cells expressing high levels of GITR remained unchanged after LT administration (Figure 1A).

Since LT promotes an intense T-cell apoptosis [3, 16], we explored whether the maintenance in the number of $CD4^+GITR^{high}$ cells after LT treatment could reflect an enhanced resistance of these cells to LT induced apoptosis and/or an expansion of this cell population. For that, we explored first the effects of LT in BALB/c mice after bilateral adrenalectomy, which severely impairs LT-induced lymphocyte apoptosis [3, 16]. In these adrenalectomized mice, f.p. treatment with LT did not modify significantly the numbers of $CD4^+CD69^+$, $CD4^+$CTLA-4^+, $CD4^+CD45RB^{-/low}$ or $CD4^+CD62L^-$ cells and slightly reduced the number of $CD4^+CD25^+$ cells (Figure 1A). In contrast, the number of $CD4^+CD25^-GITR^{high}$ cells was increased in these mice (Figure 1A). In addition, no differences in the susceptibility of peripheral $CD4^+$ T cells to f.p. LT-induced apoptosis were found between Sv129-$GITR^{-/-}$ mice and wild type littermates [nº of spleen $CD4^+$ T cells ($x10^6$) in; Sv129 wild type mice receiving PBS: 16.7 ± 1.3, or 1 μg of LT: 5.7 ± 0.4 (n= 4); in Sv129-$GITR^{-/-}$ mice receiving PBS: 13.6 ± 4.6, or 1 μg of LT: 4.3 ± 0.8 (n= 3)]. Induction of GITR expression was observed in $CD4^+CD25^-$ $FoxP3^-$ T cells but not in $CD4^+CD25^+FoxP3^+$ Tregs (Figure 1B). The expansion of $CD4^+CD25^-GITR^{high}$ cells in adrenalectomized mice was evident as early as 24 hrs after LT administration, returning to basal levels 10 days later (Figure 1C) and was dose

dependent (Figure 1D). A similar increase in $CD4^+CD25^-GITR^{high}$ cells was observed 72 hrs after f.p. administration of LT in C57BL/6 (B6) Tg mice overexpressing in T cells both a dominant negative form of FADD (FADD-DN) and human Bcl-2 (hBcl-2) (Figure 1E) and which are refractory to the LT-induced apoptosis of mature T cells [16]. LT also promoted the expression of GITR in inguinal and mesenteric lymph node cells and purified $CD4^+CD25^-$ spleen T cells stimulated *in vitro* with LT during 24 hrs (Table 1). Finally, f.p. injection of LT into bilateral adrenalectomized BALB/c mice increased the number of $CD8^+GITR^{high}$ cells but not of $B220^+GITR^{high}$ cells (Figure 1F).

To investigate whether the expansion of $CD4^+CD25^-GITR^{high}$ cells in the LT treated mice was due to the induction of GITR expression in naïve $CD4^+$ T cells or to the proliferation of pre-existing $CD4^+CD25^-GITR^{high}$ cells, bilateral adrenalectomized BALB/c mice were treated with BrdU prior and after the f.p. injection of LT. 72 hrs after toxin administration the percentage of spleen $CD4^+BrdU^+$ cells was similar to that found in untreated mice (Figure 2). In addition, LT did not promote an increase in $CD8^+BrdU^+$ and $B220^+BrdU^+$ cells in these mice (Figure 2).

We further investigated the capacity of LT to induce GITR expression in lymphocytes after mucosal administration. Intragastric (i.g.) administration of 10 or 50 μg of LT into BALB/c mice failed to promote GITR expression 48 hrs later in $CD4^+CD25^-$ T cells from both the Peyer's Patches (% of $CD4^+GITR^+$ T cells in, untreated: 19.7 ± 2.1%; treated with 10 μg of LT: 18.6 ± 1.3%; treated with 50 μg of LT: 25.1 ± 4.1%; n=5; $p>0.08$ in all cases) and the draining mesenteric lymph nodes (% of $CD4^+GITR^+$ T cells in, untreated: 18.9 ± 4.6%; treated with 10 μg of LT: 16.1 ± 1.9%; treated with 50 μg of LT: 21.7 ± 4.5%; n=5; $p>0.08$ in all cases), even after

treatment with Neomycin before (48 hrs) and after toxin administration (data not shown). Similarly, LT (10 μg) did not induce GITR in $CD4^+CD25^-$ T cells from the draining mediastinal lymph nodes when administered intranasally (i.n., data not shown). In contrast, *in vitro* stimulation of Peyer´s Patches cells with LT during 24 hrs strongly induced the expression of GITR on $CD4^+CD25^-$ T cells at levels comparable to those observed in *in vitro* LT-stimulated $CD4^+CD25^-$ T cells from mesenteric lymph nodes (Table 1).

T-cell induction of GITR by LT requires its enzymatic activity and is specific of this adjuvant.

To explore whether the enzymatic activity of LT was required to induce the expression of GITR in $CD4^+$ T cells, bilateral adrenalectomized BALB/c mice were injected into the f.p. with 1 or 30 μg of either the enzymatically inactive or the partially active LTK63 and LTR72 mutants, respectively [4, 5]. At the higher dose, but not with the lower dose, of the toxins the partially active LTR72 mutant, but not the fully detoxified LTK63 mutant, was able to induce GITR expression in naïve $CD4^+CD25^-$ T cells (Figure 3 and data not shown).

We then investigated the capacity of different adjuvants/immunostimulators such as CFA, aluminium hydroxide (Alum) and MF59 or LPS, to regulate GITR expression in $CD4^+$ T cells from BALB/c mice. None of these adjuvants/immunostimulators administered i.p. modified significantly the numbers of $CD4^+CD25^-GITR^{high}$ cells in the spleen of these mice (Figure 3).

Systemic administration of LT blocks the induction of tolerance by a GITR-dependent mechanism.

Pre-treatment of adult mice with monomeric deaggregated human gammaglobulin (DHGG) induces immunological tolerance against the immunogenic and aggregated HGG (AHGG) in murine mature lymphocytes [20]. Using this experimental model we first compared the capacity of LT and LTK63 to interfere with the induction of immunological tolerance. BALB/c mice injected i.p. on day 0 with 3 mg of DHGG and boosted 10 days later with 400 μg of AHGG, failed to produce IgG anti-HGG Abs 7 days after AHGG immunization (Figure 4A). As previously reported [20], mice receiving LPS at the time of DHGG treatment produced high levels of IgG anti-HGG Abs (Figure 4A). A similar rupture of tolerance was observed when DHGG-injected BALB/c mice received 1 μg of LT into the f.p., but not 5 μg of LTK63 (Figure 4A). In contrast, the f.p. treatment with 0.1 μg of LT failed to block the induction of tolerance to HGG (data not shown), in agreement with the absence of induction of GITR expression in T cells (Figure 1D). The induction of tolerance in this model was not due to the activity of Tregs. Thus, DHGG-tolerized BALB/c mice depleted in Tregs after treatment with an anti-CD25 mAb, did not produce significant levels of IgG anti-HGG Abs after AHGG immunization, in contrast with non-tolerized but AHGG immunized BALB/c mice depleted in Tregs (Figure 4A).

We then explored the role of the induction of GITR in $CD4^+$ T cells in the mechanism of systemic adjuvanticity of LT. The engagement of GITR with the agonistic anti-GITR mAb DTA-1 [18] at the time of DHGG injection blocked the induction of tolerance to HGG and these mice exhibited increased serum levels of IgG anti-HGG Abs (Figure 4A). Interestingly, f.p. administration of LT into Sv129-$GITR^{-/-}$

mice failed to block the induction of tolerance to HGG, as efficiently as observed in wild type controls; these mice had reduced levels of IgG anti-HGG Abs that were at titres close, although slightly higher, to those found in DHGG-AHGG tolerized controls (Figure 4B).

LT and LTK63 exhibit similar systemic but not mucosal adjuvanticity in GITR$^{-/-}$ mice.

In view of the potential importance of GITR induction in CD4^{+} T cells in the systemic adjuvanticity of LT associated with its enzymatic activity, we have explored whether in an immunization protocol where LT and LTK63 trigger an immune response to a co-administered antigen, the systemic adjuvanticity of both toxins will be similar in GITR$^{-/-}$ mice. Sv129-GITR$^{-/-}$ and wild type mice were primed and boosted 20 days later i.p. with OVA using LT, LTK63 or CFA/IFA as adjuvants. According to previous studies [4, 5], 15 days after the second immunization the levels of IgG anti-OVA Abs were higher in wild type mice immunized with OVA-LT than in wild type mice immunized with OVA-LTK-63 (Figure 5A). However, an important reduction in the levels of IgG anti-OVA Abs was observed in Sv129-GITR$^{-/-}$ mice immunized i.p. with OVA-LT that were similar to those found in both groups of mice immunized i.p. with OVA-LTK63 (Figure 5A). As a control, no differences in the levels of IgG anti-OVA Abs were observed between wild type and Sv129-GITR$^{-/-}$ mice when CFA/IFA was used as adjuvant (Figure 5A). The decreased IgG anti-OVA Ab production observed in LT-OVA immunized Sv129-GITR$^{-/-}$ mice was associated with a selective reduction in the levels of IgG2a anti-OVA Abs ($p<0.01$), being the titres of IgG1 and IgG2b anti-OVA Abs similar between both groups of animals ($p>0.05$ in both cases) (Figure 5B).

Finally, similar levels of circulating IgA anti-OVA Abs were measured in both groups of mice (data not shown).

To explore the mechanisms accounting for the selective reduction of IgG2a anti-OVA Abs in LT-treated Sv129-GITR$^{-/-}$ mice, the expression of IFNγ and IL-4 in the spleen was compared between bilateral adrenalectomized Sv129-GITR$^{-/-}$ and wild type mice treated i.p. with 1 μg of LT by real time quantitative RT-PCR. An increase in IFNγ and IL-4 mRNA transcripts was observed in the spleen of Sv129 wild type mice 5 days after LT administration (Figure 5C). The expression of IL-4 was also enhanced in the spleen of LT-treated Sv129-GITR$^{-/-}$ mice but the levels of IFNγ mRNA were essentially identical to those of PBS treated Sv129-GITR$^{-/-}$ controls (Figure 5C).

We finally compared the mucosal adjuvanticity of LT and LTK63 between wild type and Sv129-GITR$^{-/-}$ mice immunized and boosted i.n. with OVA dissolved in either PBS, 10 μg of LT or LTK63. No differences in the levels of circulating IgG, IgA or the different IgG subclasses (Figure 6 and data not shown) anti-OVA Abs were observed between LT-OVA i.n immunized wild type and Sv129-GITR$^{-/-}$ mice 14 days after the boost. Serum levels of IgG anti-OVA Abs were also similar between wild type and Sv129-GITR$^{-/-}$ mice immunized i.n. with LTK63-OVA but significantly lower ($p<0.001$) than those observed in mice receiving LT as adjuvant (Figure 5B).

Discussion

The unique property of LT to act as a potent mucosal and systemic adjuvant has stimulated many studies aimed at understanding its mechanism of action [21]). Here, we demonstrate that LT induces the expression of GITR, but not other activation markers,

on T cells when administered systemically by a mechanism that requires the enzymatic activity of the toxin and is independent of endogenous glucocorticoids. This effect seems specific of LT since is not observed with other commonly used adjuvants or immune stimulators. The induction of GITR expression in $CD4^+$ T cells accounts for the systemic adjuvanticity of LT which is associated to its enzymatic activity. Finally, the inability of LT to promote GITR expression in Peyer´s Patches T cells after its mucosal administration and the fact that wild type and $GITR^{-/-}$ mice respond equally to i.n. administered antigens using either LT or LTK63 as adjuvants, clearly indicates that the mechanisms of adjuvanticity of these toxins are highly influenced by their route of administration.

We describe a novel mechanism of systemic adjuvanticity of LT linked with the capacity of the toxin to induce the expression of key costimulatory molecules, such as GITR, in $CD4^+$ T cells. Since LT causes apoptosis of mature lymphocytes, the accumulation of $CD4^+GITR^{high}$ cells after toxin administration may reflect a particular resistance of this cell population to cell death signals, as it has been suggested previously [22]. It is theoretically possible that in situations where the ability of LT to promote T cell apoptosis is inhibited, the relative proportion of $CD4^+GITR^{high}$ and $CD4^+GITR^{low}$ cells will be close to that observed in untreated mice and that such resistance will be not observed in mice deficient in GITR. However, although we can not totally exclude this possibility, our results demonstrate a similar susceptibility of $CD4^+$ T cells from Sv129 wild type and $GITR^{-/-}$ mice to LT-induced apoptosis and an expansion of $CD4^+GITR^{high}$ cells in both bilateral adrenalectomized BALB/c mice and B6 mice overexpressing in T cells FADD-DN and hBcl-2 transgenes, which are largely protected against LT-induced apoptosis of mature T cells [3, 16].

In view of the role of GITR in T cells, GITR signalling may contribute to the adjuvanticity of LT by acting as an activation-induced costimulatory signal in $CD4^+CD25^-$ T cells that increase their TCR-induced proliferation and cytokine production [17]. Signalling through GITR may also inhibit the suppressive capacity of Tregs [18] or may render $CD4^+CD25^-$ T cells more resistant to the Treg-mediated suppression [17], also contributing in that way to the immunostimulatory capacity of LT. However, against this last possibility is the fact that LT interferes with the establishment of lymphocyte tolerance in a model in which such tolerance induction is independent of Treg activity. Alternatively, the induction of GITR expression in $CD4^+$ T cells may influence the adjuvanticity of LT by regulating the activity of DC. GITR is activated by its ligand (GITRL), which is expressed in APCs [17]. Although our preliminary observations fail to show changes in the expression of GITRL on APC after LT administration (unpublished observations), it is also possible that GITRL, interacting with the upregulated GITR receptor in T cells, may deliver signals to APC by means of its cytoplasmic domain [19]. In this regard, DC cocultured with activated $CD4^+$ T cells from $GITR^{-/-}$ mice and stimulated with heat-inactivated *Candida albicans* produce high amounts of IL-12 [23]. However this Th1 polarization observed in $GITR^{-/-}$ mice during *Candida albicans* immune responses clearly contrasts with the reduction in the levels of circulating IgG2a (the classical Th1 IgG subclass) anti-OVA Abs and with the absence of induction of IFNγ gene expression in the spleen of these LT-treated $GITR^{-/-}$ mice. Whether the different nature of these two antigens may explain these apparently conflicting observations is at present unknown.

LT retains, although at decreased levels, a prominent systemic immunostimulatory capacity in $GITR^{-/-}$ mice that is similar in intensity to the adjuvanticity of LTK63 in wild type and $GITR^{-/-}$ mice. Thus, other still unknown mechanisms have to be involved in the capacity of LT to stimulate the immune system, mechanisms which are very likely shared by the LTK63 mutant, and which may be linked to the holotoxin structure or to their receptor binding capacity. In particular, GM1 crosslinking induces the activation of T cells and the B subunit of CT or LT are capable of substituting for costimulation during T cell activation or to elicit humoral immune responses in mice, respectively [24-27]. However, the B subunit of LT promotes the induction of immunological tolerance to antigens co-administered orally [28], suggesting that the crosslinking of GM1 by LT or LTK63 may contribute to the adjuvanticity of these toxins only in certain experimental conditions.

Unlike the systemic route, the mucosal administration of LT does not induce the expression of GITR on T cells in both Peyer's Patches and draining mesenteric lymph nodes. In agreement with these results, no differences in the LT adjuvanticity are observed between wild type and $GITR^{-/-}$ mice. Since LT promotes a rapid and strong upregulation of GITR expression in Peyer's Patches $CD4^{+}CD25^{-}$ T cells in vitro, the effects observed in vivo are unrelated to the presence of intrinsic defects in these mucosal T cells. Although doses of LT 10 to 50 times higher than those used systemically and administered with Neomycin fail to induce GITR expression in T cells, such absence of GITR induction after mucosal delivery of LT may be due to differences in the availability of the toxin when given parenterally or mucosally secondary to an enhanced degradation of the toxin. However, the fact that these high doses of LT administered i.n. have a strong immunostimulatory activity against co-administered

antigens, clearly indicates that the mucosal adjuvanticity of LT is unrelated to GITR. Thus, our results provide evidences that the mechanisms of adjuvanticity of LT are highly influenced by the route of administration of the toxin. Other groups have demonstrated that the i.g. administration of CT enhances the expression of B7-2 molecule on B cells and macrophages and that this induction is critical for the mucosal adjuvanticity of the toxin [29]. However, it remains unclear whether a similar upregulation of B7-2 expression is observed after mucosal or systemic administration of LT.

In summary, here we have demonstrated that distinct mechanisms can be in place when adjuvants such as LT are administered parenterally or mucosally. In fact, the systemic adjuvanticity of LT which is linked to its enzymatic activity is mediated through the regulation of GITR expression in $CD4^+$ T cells. The induction of Bell's palsy in some healthy subjects receiving i.n. LT or LT mutants containing vaccines [8, 9] supports the possibility of using these adjuvants via the parenteral route. In this light, our study provides insights into the understanding of immunomodulatory properties of LT and related toxins that may lead to the harnessing of such activities in safer forms.

Materials and Methods

Mice and surgery.

BALB/c mice were obtained from Harland Iberica (Barcelona, Spain). B6-*prLck.hbcl-2* Tg mice were obtained by backcrossing C3H/HeN.*Lck.hbcl-2* Tg mice (Jackson Laboratories, Bar Harbor, ME) with B6 mice. B6. *prLck.hbcl-2* Tg mice were crossed with B6 Tg mice overexpressing FADD-DN in T cells [30], generously provided by Dr Andreas Strasser, (The Walter and Eliza Hall Institute of Medical

Research, Australia), to obtain double-Tg mice. Sv129-GITR$^{-/-}$ mice were generated by homologous recombination as previously described [31].

Adrenal glands were removed as described [3]. LT toxin was administered 2 days after surgery.

All experiments were performed in 6-8 wk old animals and approved by the Universidad de Cantabria Institutional Laboratory Animal Care and Use Committee.

Adjuvants and immunization protocols.

LT and their mutants, MF59 and Alum were obtained from Novartis Vaccines (Siena, Italy). The CFA and IFA were purchased from Sigma (St Louis, MO). The doses of LT and LT mutants used here were chosen according to our previous studies [3, 16]. For i.g. administration of toxins (10 or 50 μg of LT or 30 μg of LTK63), animals received previously 200 μl of 1M carbonate-bicarbonate buffer and 100 μg of omeprazole (Sigma) and were maintained under Neomycin treatment (SALVAT Laboratories, Barcelona, Spain), administered in the drinking water at a concentration of 5 mg/l).

DHGG and AHGG were prepared from HGG (Baxter S.L., Valencia, Spain) as described previously [20]. Mice were treated i.p. (BALB/c mice) or i.v. (Sv129 mice) with 3 mg of DHGG and boosted i.p. 10 days later with 400 μg of AHGG dissolved in saline (BALB/c mice) or emulsified in CFA (Sv129 mice). In some experiments, 50 μg of LPS, 1 or 0.1 μg of LT or 5 μg of LTK63 were injected i.p. 3 hrs after DHGG

injection. The presence of IgG anti-HGG Abs in sera was measured by ELISA 7 days after the AHGG boost, as described [20].

In some experiments, Sv129-GITR$^{-/-}$ mice and wild type littermates were immunized i.p. with 100 μg of OVA together with either 1 μg of LT or 5 μg of LTK63. A similar boost was performed 20 days later. As a control, mice were immunized with 100 μg of OVA-CFA and boosted with 100 μg of OVA-IFA 20 days later. The mucosal adjuvanticity of toxins were tested by immunizing i.n. Sv129-GITR$^{-/-}$ mice and wild type littermates on days 0 and 21 with 10 μg of toxins and 10 μg of OVA, or with antigen alone. In all experiments, the titres of IgA, IgG and IgG subclass specific anti-OVA Abs in sera were measured by ELISA 15 days after the boost, as described [5].

Induction of apoptosis by LT.

Sv129-GITR$^{-/-}$ mice and wild type littermates were injected into the f.p. with 1 μg of LT and the induction of apoptosis of CD4^{+} T cells was evaluated 72 hrs later as described previously [3, 16].

Flow cytometry studies.

Frequencies of lymphocyte populations were evaluated in the spleen, mesenteric lymph nodes or Peyer's Patches by flow cytometry 48-72 hrs after the administration of toxins using conjugated mAbs (Becton Dickinson Biosciences, Madrid, Spain). Cells were analyzed using a FACScalibur flow cytometer using the Cell Quest Pro software (Becton Dickinson).

CD4^{+}CD25^{-} T cell purification and *in vitro* assay.

Purified $CD4^+CD25^-$ spleen cells, using the mouse $CD4^+$ isolation kit (Miltenyi Biotec, Madrid, Spain)or total Peyer's Patches, inguinal or mesenteric lymph node cells (5 $x10^5$ cells/well) from 2 month old BALB/c were either non-stimulated (medium) or stimulated in vitro with LT (0.5 μg/ml) during 24h. GITR expression on viable cells was evaluated by flow cytometry.

***In vivo* proliferation assay.**

Mice received an i.p. injection of 1 mg of BrdU (Sigma) prior to the treatment with 1 μg LT and maintained with BrdU in the drinking water (0.8 mg/ml) during the following three days. The incorporation of BrdU in mature B and T spleen cells was evaluated by flow cytometry 72 hrs after LT administration, as described previously [32].

Treatment with mAbs.

For $CD4^+CD25^+$ T cell depletion, mice received one daily i.p. injection of 0.2 mg of anti-CD25 mAb (clone PC61) during three days one week before the immunization with AHGG or DHGG. The efficiency of the treatment was confirmed by flow cytometry. In some experiments, mice received i.p. 1 mg of an agonistic anti-GITR mAb (DTA-1) [18], generously provided by Dr Simon Sakaguchi (Kyoto University, Kyoto, Japan), at the time of DHGG tolerization.

Real time RT-PCR assays.

Total RNA was obtained from the spleen of bilateral adrenalectomized Sv129-$GITR^{-/-}$ mice and wild type littermates 5 days after i.p. injection of 1 μg of LT or PBS and used for cDNA synthesis with a RT-PCR kit (Fermentas, Quimigen S.L., Madrid).

Quantitative, real time PCR was conducted on a MX-3000P Stratagene instrument (La Jolla, CA) using specific TaqMan POMC expression assays (Applied Biosystems, Life Technologies). Results (in triplicate) were normalized to GAPDH expression. Data were expressed as mean fold change relative to control samples (n= 5 mice/group).

Statistical analysis.

Statistical analysis was performed using the two-tailed Student´s *t* test or the Mann-Whitney test. Probability values <0.05 were considered significant.

Acknowledgements

We thank Maria Aramburu, and Natalia Cobo for technical assistance. This work was supported by grants from the Ministerio de Educación y Ciencia, Spain to RM (SAF2008-02042) and JM (SAF2006-12520-C02-02 and BFU2009-07206), from the Fundación Marqués de Valdecilla, Spain to JM (API-07/02), by a research agreement sponsored by Novartis Vaccines and Diagnostics (to RM and JM), by grant REDINREN RD06/0016 from the “Instituto de Salud Carlos III” to RM and by grant no LSHP-CT-2003–503240 (MUVAPRED, Mucosal Vaccines for Poverty Related Diseases) from the European Commission" to GDG and RR.

Conflict of interest

With the exception of Rino Rappuoli and Giuseppe de Giudice, that are full time employees of Novartis Vaccines and Diagnostics, Siena, Italy, the authors have no conflicting financial interests.

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Figure Legends

Figure 1. Transient T-cell induction of GITR expression after systemic injection of LT. A) Expression of activation markers in $CD4^+$ T cells in the spleen 3 days after f.p. injection of 1 μg of LT or PBS into normal or bilateral adrenalectomized (*Adrx*) BALB/c mice. The reduction (↓) and increase (↑) indexes after LT treatment are indicated. B) Induction of GITR expression on $CD4^+CD25^-FoxP3^-$ but not on $CD4^+CD25^+FoxP3^+$ T cells from bilateral adrenalectomized BALB/c mice. C) Kinetics of GITR induction in $CD4^+$ T cells in the spleen of bilateral adrenalectomized BALB/c mice after f.p. injection of LT. D) Dose dependent induction of GITR expression on $CD4^+$ T cells in the spleen of bilateral adrenalectomized BALB/c mice 72 hrs after f.p. injection of LT. E) Number of $CD4^+GITR^{high}$ T cells in the spleen of B6.*Lck.hbcl-2*/FADD-DN double-Tg mice 3 days after the f.p. injection of PBS or 1 μg of LT. F) Number of $CD8^+GITR^{high}$ T and $B220^+GITR^{high}$ cells in the spleen of bilateral adrenalectomized BALB/c mice treated with PBS or with 1 μg of LT into the f.p. The results, representative of at least 2 independent experiments (n=5 mice per group), are expressed as the mean ± SEM of the number of viable cells (A, C-F) or MFI of GITR expression (**B**) of the indicated populations. Statistic differences between PBS and LT treated animals, evaluated by the Student´s *t* test, are indicated as follow: ns. non-significant, *p<0.05, **p<0.01, ***p<0.001.

Figure 2. LT fails to induce the proliferation of mature B and T cells. Percentages of $BrdU^+$-$B220^+$, $BrdU^+$-$CD4^+$ or $BrdU^+$-$CD8^+$ splenocytes in bilateral adrenalectomized BALB/c mice 3 days after f.p. administration of LT expressed as the mean ± SEM. Results are representative of 3 independent experiments (n=5 mice per

group). No statistic differences, evaluated by the Student´s *t* test, were observed between PBS and LT treated animals.

Figure 3. The LT induction of GITR in T cells requires its enzymatic activity and is specific of this adjuvant. BALB/c mice (3-5 mice/group) were injected into the f.p. with 1 μg of LT, 30 μg of LTR72 or LTK63 or i.p. with 200 μl of CFA (50% v/v), 50 μg of LPS, 1 mg of Alum or 200 μl of MF59 (50% v/v). Representative panels of 2 independent experiments show the expression of GITR in $CD4^+CD25^-$ splenocytes (solid line) measured by flow cytometry 72h later. Dotted lines represent background fluorescence. The mean ± SEM of the number of $CD4^+GITR^{high}$ cells (top) and the mean fluorescence intensity (MFI) of GITR expression (bottom) are indicated. Statistic differences between PBS and adjuvant treated animals, evaluated by the Student´s *t* test, are indicated as follow: ns. non-significant, ***$p<0.001$.

Figure 4. LT but not LTK63 blocks the induction of tolerance to HGG by a GITR-dependent mechanism. A) Two month-old BALB/c mice were tolerized with DHGG and immunized i.p. with AHGG 10 days later. Non immunized mice or animals injected i.p. with AHGG were used as controls. Tolerized mice were either untreated or treated with LPS, LT or LTK63 i.p. at the time of DHGG injection or treated with either a cytotoxic anti-CD25 mAb or an agonistic anti-GITR mAb (DTA-1). **B)** Two month-old Sv129-$GITR^{-/-}$ mice or wild type littermates were tolerized with DHGG and treated or not with 1 μg of LT 10 days before the immunization with AHGG-CFA. The presence of circulating IgG anti-HGG Abs was evaluated 7 days later by ELISA. Values of individual mice are expressed in TU. Bars represent the mean value of each examination. Statistic differences between PBS and LT treated animals, evaluated by

the Mann-Whitney test, are indicated as follow: ns. non-significant, *p<0.05, **p<0.01, ***p<0.001. Results are representative of 3 independent experiments.

Figure 5. The expression of GITR in T cells modulates the systemic adjuvanticity of LT. A) Two month old Sv129-GITR$^{-/-}$ and wild type littermates were immunized i.p. and boosted 20 days later with 100 µg of OVA dissolved in saline or coadministered with either 1 µg of LT or 5 µg of LTK63. Control mice were immunized with 100 µg of OVA emulsified in CFA and boosted with 100 µg of OVA emulsified in IFA 20 days later. Bars represent the mean ± SEM of the titres of IgG anti-OVA Abs in sera 15 days after the boost. Results are representative of 2 independent experiments. **B)** Levels of IgG1, IgG2a and IgG2b anti-OVA Abs in the same sera than A. Values of individual mice are expressed in TU. Bars represent the mean value of each examination. Statistic differences between the groups, evaluated by the Mann-Whitney test, are indicated as follow: ns. non-significant, **p<0.01. **C)** Expression of IFNγ and IL-4 mRNA in the spleen of bilateral adrenalectomized BALB/c mice 5 days after f.p. injection of 1 µg of LT evaluated by real time quantitative RT-PCR. Results for each cytokine are normalized to GAPDH expression an expressed as the mean ± SD of 5 mice per group. Statistic differences between PBS and LT treated animals, evaluated by the Student´s *t* test, are indicated as follow: *p<0.05, **p<0.01.

Figure 6. The expression of GITR in T cells does not influence the mucosal adjuvanticity of LT. A) Two month old Sv129-GITR$^{-/-}$ and wild type littermates were immunized and boosted 21 days later i.n. with 10 µg of OVA dissolved in PBS or coadministered with either 10 µg of LT or LTK63. The levels of circulating IgG anti-OVA Abs were evaluated 15 days after the boost. Values of individual mice are expressed in TU. Bars represent the mean value of each examination. Statistic

differences between the groups, evaluated by the Mann-Whitney test, are indicated as follow: ns. non-significant, ***$p<0.001$.

Table 1. *In vitro* induction of GITR expression on $CD4^+$ T cells by LT.

	% of $CD4^{\pm}GITR^{\pm}$		MFI of GITR	
	Medium	LT	Medium	LT
Purified $CD4^+CD25^-$ cells	18.1 ± 0.9	82.8 ± 6.1	11.2 ± 0.6	33.8 ± 1.8
Inguinal lymph node cells	16.6 ± 2.4	75.2 ± 3.5	14.9 ± 0.4	31.8 ± 2.4
Mesenteric lymph node cells	18.3 ± 1.2	76.8 ± 4.8	10.8 ± 0.3	37.9 ± 0.6
Peyer's Patches cells	17.7 ± 2.4	73.2 ± 1.7	14.1 ± 1.1	33.5 ± 2.1

The indicated cells (5 $x10^5$ cells/well) from 2 month old BALB/c were either non-stimulated (medium) or stimulated in vitro with LT (0.5 μg/ml) during 24h. Results are expressed as the mean ± SD of the percentage of $GITR^{high}$ cells and the MFI of GITR expression in viable $CD4^+CD25^-$ cells in triplicate cultures, evaluated by flow cytometry.

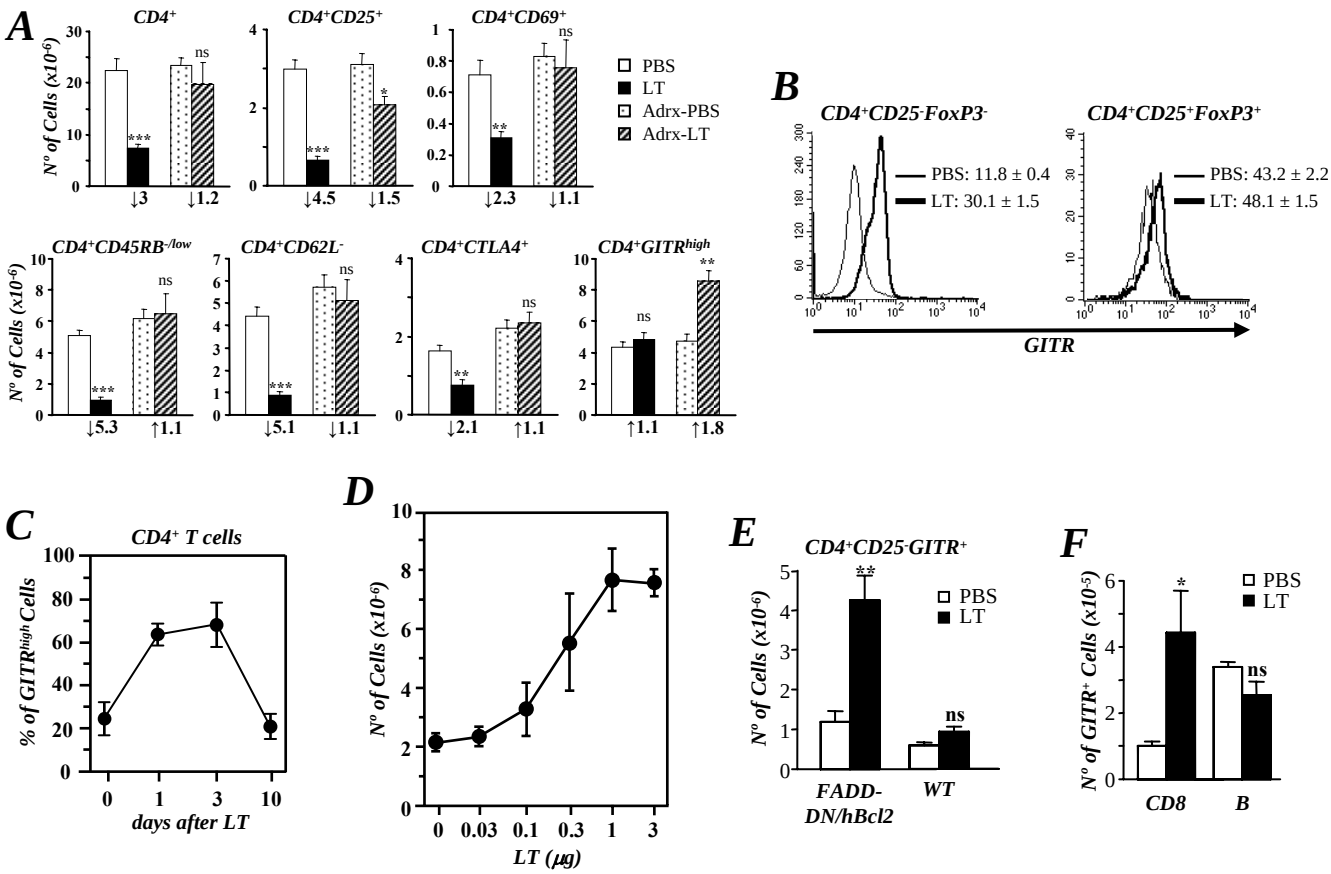

A
CD4+
CD4+CD25+
CD4+CD69+
Nº of Cells (x10-6)
PBS
LT
Adrx-PBS
Adrx-LT
ns
↓3
↓1.2
↓4.5
↓1.5
↓2.3
↓1.1
CD4+CD45RB-/low
CD4+CD62L-
CD4+CTLA4+
CD4+GITRhigh
↓5.3
↑1.1
↓5.1
↓1.1
↓2.1
↑1.1
↑1.1
↑1.8
B
CD4+CD25-FoxP3-
CD4+CD25+FoxP3+
PBS: 11.8 ± 0.4
LT: 30.1 ± 1.5
PBS: 43.2 ± 2.2
LT: 48.1 ± 1.5
GITR
C
CD4+ T cells
% of GITRhigh Cells
days after LT
D
Nº of Cells (x10-6)
LT (μg)
E
CD4+CD25-GITR+
Nº of Cells (x10-6)
FADD-DN/hBcl2
WT
F
Nº of GITR+ Cells (x10-5)
CD8
B

Figure 1

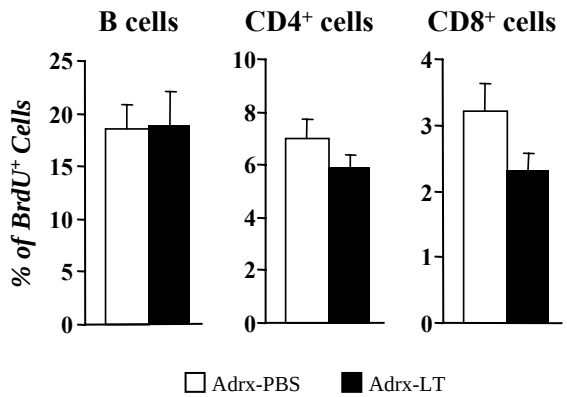

B cells
CD4+ cells
CD8+ cells
% of BrdU+ Cells
25
20
15
10
5
0
10
8
6
4
2
0
4
3
2
1
0
Adrx-PBS
Adrx-LT

Figure 2

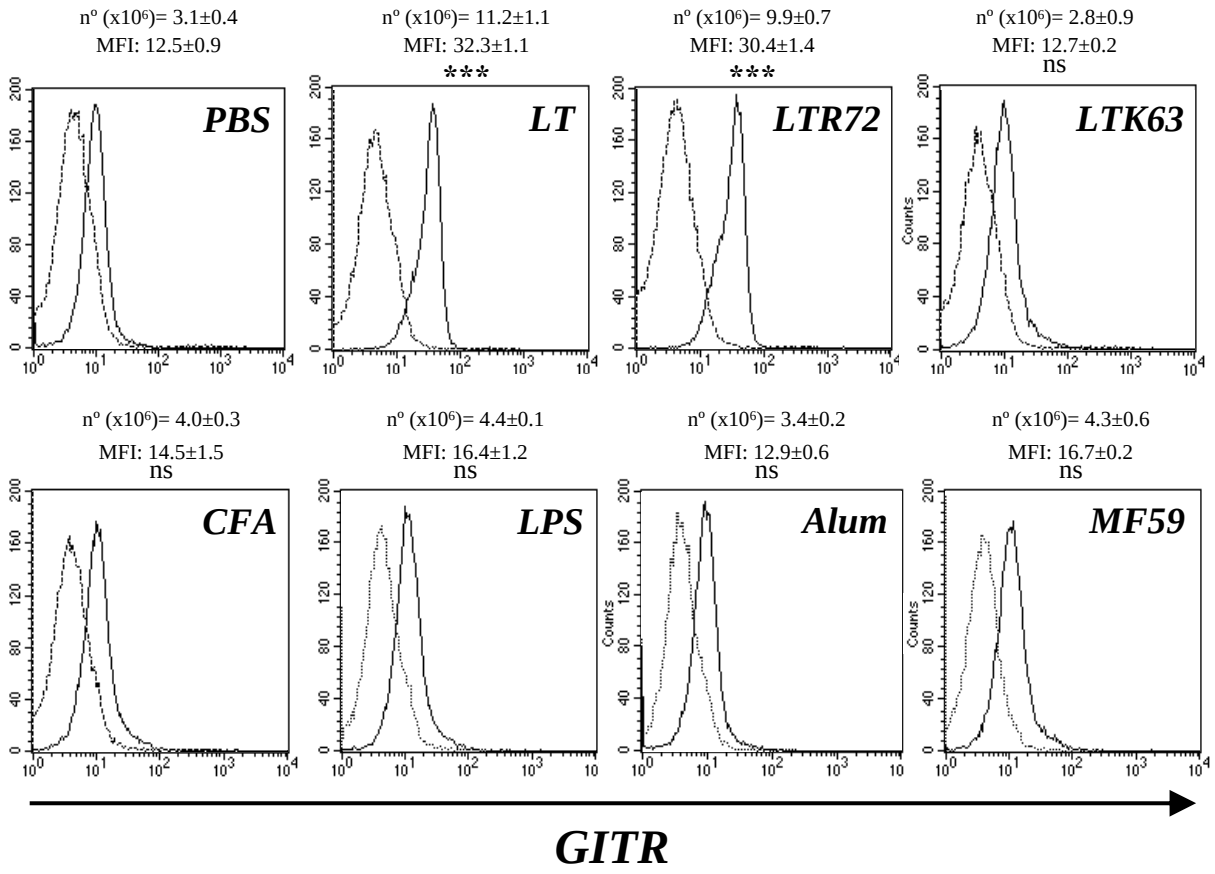

nº (x10⁶)= 3.1±0.4
MFI: 12.5±0.9
PBS
nº (x10⁶)= 11.2±1.1
MFI: 32.3±1.1
***
LT
nº (x10⁶)= 9.9±0.7
MFI: 30.4±1.4
***
LTR72
nº (x10⁶)= 2.8±0.9
MFI: 12.7±0.2
ns
LTK63
nº (x10⁶)= 4.0±0.3
MFI: 14.5±1.5
ns
CFA
nº (x10⁶)= 4.4±0.1
MFI: 16.4±1.2
ns
LPS
nº (x10⁶)= 3.4±0.2
MFI: 12.9±0.6
ns
Alum
nº (x10⁶)= 4.3±0.6
MFI: 16.7±0.2
ns
MF59
Counts
GITR

Figure 3

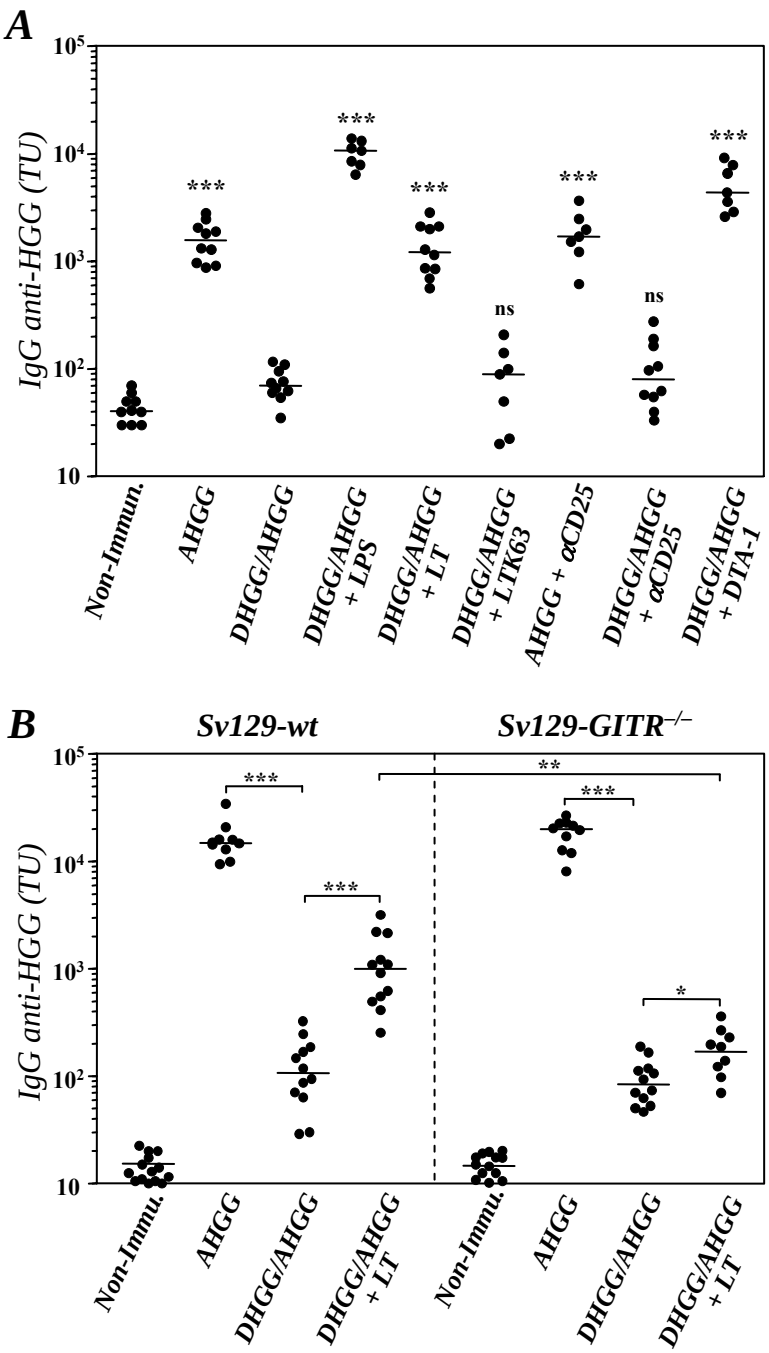
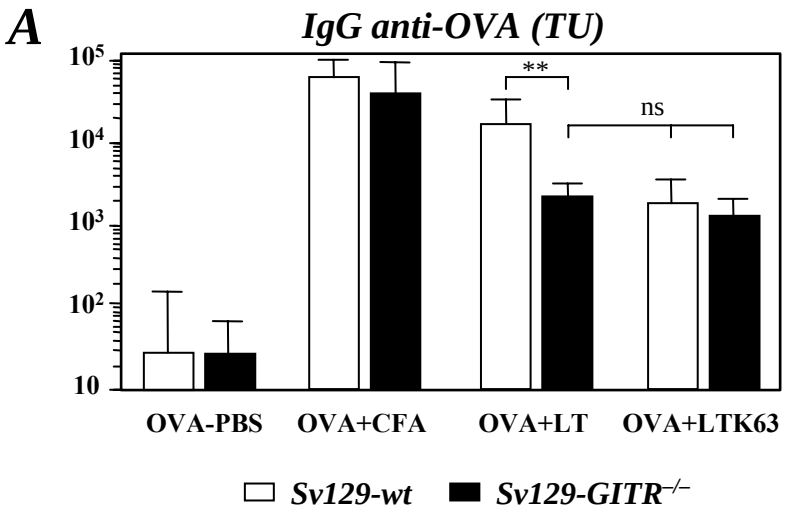
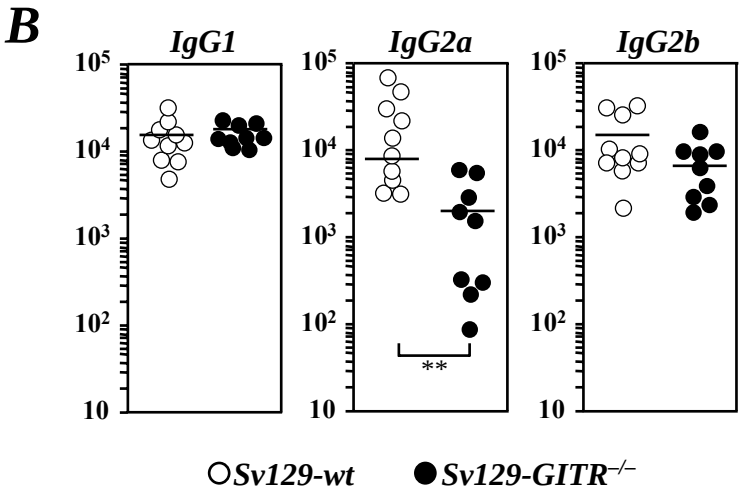

A
IgG anti-HGG (TU)
10^5
10^4
10^3
10^2
10
***
***
***
***
ns
***
ns
***
Non-Immun.
AHGG
DHGG/AHGG
DHGG/AHGG + LPS
DHGG/AHGG + LT
DHGG/AHGG + LTK63
AHGG + αCD25
DHGG/AHGG + αCD25
DHGG/AHGG + DTA-1
B
Sv129-wt
Sv129-GITR−/−
IgG anti-HGG (TU)
10^5
10^4
10^3
10^2
10
***
***
**
***
*
Non-Immu.
AHGG
DHGG/AHGG
DHGG/AHGG + LT
Non-Immu.
AHGG
DHGG/AHGG
DHGG/AHGG + LT

Figure 4


A
IgG anti-OVA (TU)
10^5
10^4
10^3
10^2
10
**
ns
OVA-PBS
OVA+CFA
OVA+LT
OVA+LTK63
Sv129-wt
Sv129-GITR−/−


B
IgG1
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IgG2b
10^5
10^4
10^3
10^2
10
**
Sv129-wt
Sv129-GITR−/−

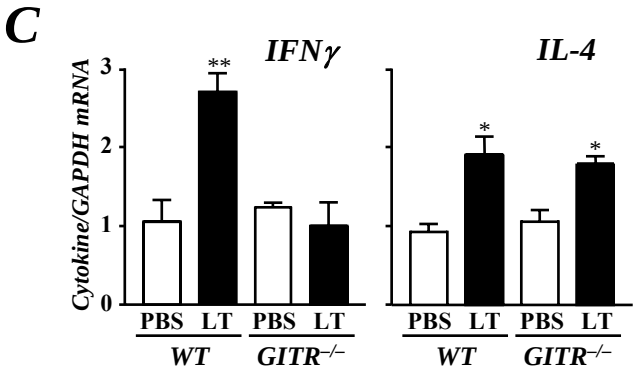
C
IFNγ
IL-4
Cytokine/GAPDH mRNA
3
2
1
0
**
*
*
PBS
LT
PBS
LT
WT
GITR−/−
PBS
LT
PBS
LT
WT
GITR−/−

Figure 5

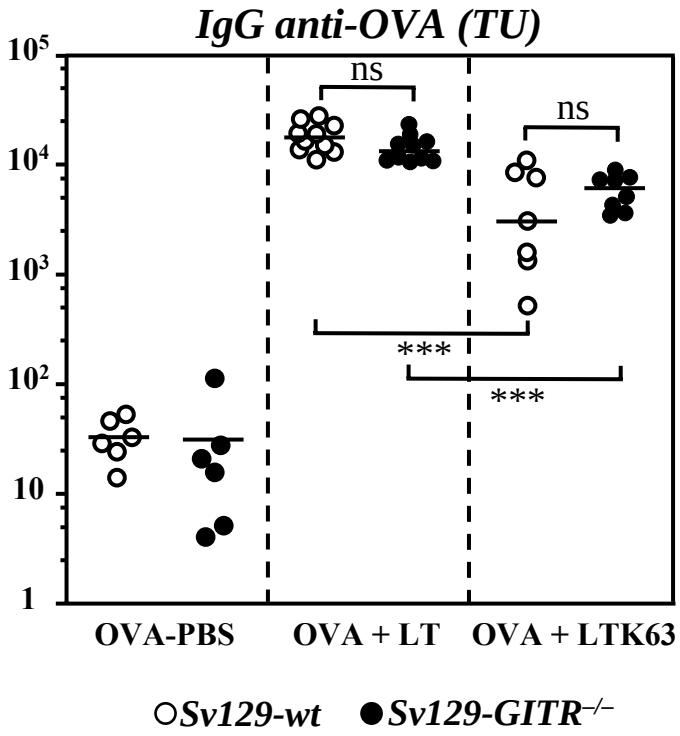
IgG anti-OVA (TU)
10^5
10^4
10^3
10^2
10
1
ns
ns
***
***
OVA-PBS
OVA + LT
OVA + LTK63
○Sv129-wt ●Sv129-GITR−/−

Figure 6